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Review

Molecular Control of Induced Pluripotency

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Deciphering the mechanisms of epigenetic reprogramming provides fundamental insights into cell fate decisions, which in turn reveal strategies to make the reprogramming process increasingly efficient. Here we review recent advances in epigenetic reprogramming to pluripotency with a focus on the principal molecular regulators. We examine the trajectories connecting somatic and pluripotent cells, genetic and chemical methodologies for inducing pluripotency, the role of endogenous master transcription factors in establishing the pluripotent state, and functional interactions between reprogramming factors and epigenetic regulators. Defining the crosstalk among the diverse molecular actors implicated in cellular reprogramming presents a major challenge for future inquiry.

Introduction

Since the initial discovery of induced pluripotent stem cells (iPSCs) (Takahashi and Yamanaka, 2006), the process of somatic cell reprogramming has been a source of fascination for the scientific community and general public alike. Cellular reprogramming is inherently an epigenetic phenomenon. Heritable modifications of DNA and chromatin, such as methylation of cytosine residues and posttranslational modifications of histones, regulate gene expression patterns during mammalian development (Reik et al., 2001). Consequently, induced pluripotency involves the dynamic rearrangement of epigenetic landscapes (Apostolou and Hochedlinger, 2013). The past several years have seen concerted efforts to elucidate the mechanisms of reprogramming, coalescing around several major questions: What genes are required for successful reprogramming? Does the process of epigenetic reprogramming follow a defined order of molecular events? What are the major barriers to induced pluripotency, and can these barriers be removed through genetic or chemical intervention?

Here we present a conceptual framework for analyzing recent mechanistic advances in epigenetic reprogramming to pluripotency that distinguishes five categories of molecular actors: (1) cell surface markers and pluripotency genes that serve as signposts for discrete transitions during reprogramming; (2) transcription factors, epigenetic regulators, and noncoding RNAs that induce reprogramming; (3) small molecules and environmental stimuli that replace the Yamanaka factors or induce novel states of pluripotency; (4) endogenous master transcription factors that coordinate the establishment of pluripotency; (5) epigenetic regulators, DNA repair proteins, and components of the basal transcriptional and translational machinery that modulate the kinetics of reprogramming.

Roadmaps of Epigenetic Reprogramming: Surface Markers and Reporter Genes that Enable Kinetic Analyses

Efforts to elucidate the sequence of molecular events during reprogramming have focused on three key events: (1) the initial transcriptional and epigenetic changes resulting from the expression of ectopic reprogramming factors, (2) the transitions through intermediate states that can be redirected toward alternative somatic destinations, and (3) the activation of a self-sustaining endogenous pluripotency circuitry. The heterogeneous and asynchronous nature of reprogramming necessitates the use of cell surface markers or reporter alleles to isolate pure populations of cells at each step. Here we review how different markers have been used to lay down roadmaps of epigenetic reprogramming (Figure 1) and the extent to which these markers can predict the successful outcome of reprogramming. We also evaluate different models of the kinetics of reprogramming.

Temporal analyses of reprogramming typically distinguish subpopulations based on the disappearance of surface markers expressed in fibroblasts and emergence of surface markers specific to pluripotent cells. Further resolution can be obtained by integrating a fluorescent reporter associated with an endogenous pluripotency determinant, such as *Oct4* or *Nanog*. Initial studies using doxycycline (dox)-inducible Oct4, Sox2, Klf4, and c-Myc (collectively these are referred to as OSKM) transgenes reported that the cell surface marker alkaline phosphatase (AP) was activated prior to stage-specific embryonic antigen 1 (SSEA1), while Oct4-GFP and Nanog-GFP reporters were induced later, concomitant with the loss of transgene dependence (Brambrink et al., 2008). An accompanying study reported that the surface antigen Thy1, which is highly expressed in fibroblasts, was downregulated prior to the onset of SSEA1 expression (Stadtfeld et al., 2008). Transcriptional and proteomic analyses of subpopulations marked by Thy1, SSEA1, and Oct4-GFP status identified two major waves of gene activity (Figure 1A) (Hansson et al., 2012; Polo et al., 2012). The first wave, between days 0 and 3, involves upregulation of genes related to cell proliferation, metabolism, and cytoskeletal organization, while developmental genes were downregulated. A second wave of gene activity occurred after day 9 in SSEA1$^+$ cells and was associated with a marked increase in pluripotency-associated genes. Gene expression analysis of Thy1$^+$ cells that persisted beyond day 3 indicated that a failure to downregulate mesenchymal genes was a signature of cells that became refractory to reprogramming shortly after dox treatment (Polo et al.,

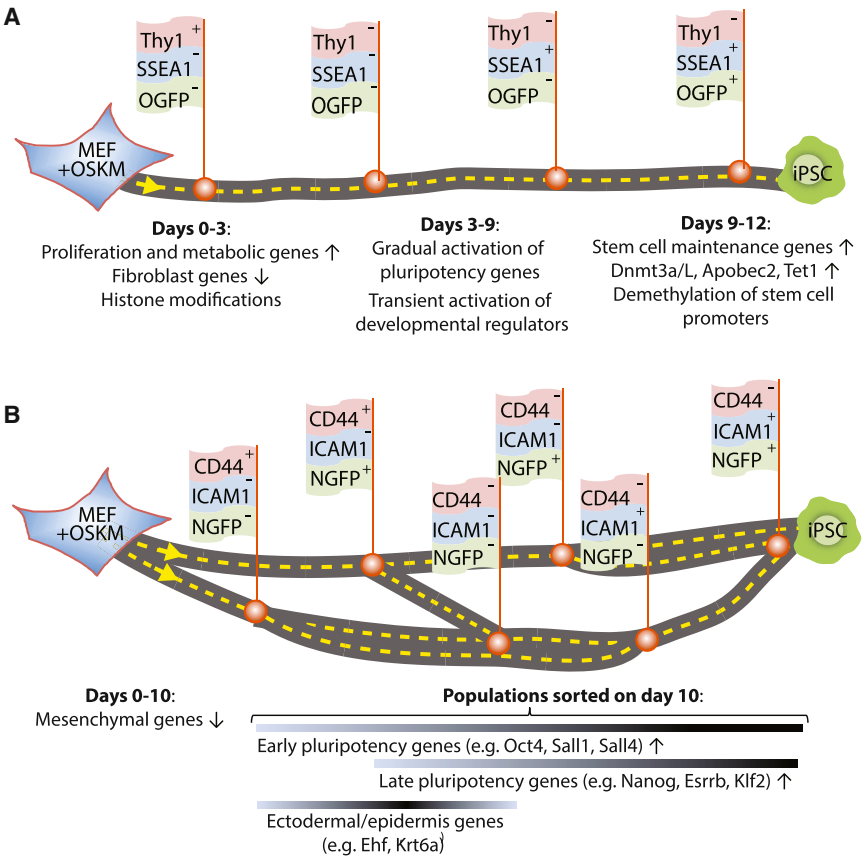


Figure 1. Roadmaps of Epigenetic Reprogramming

(A) Trajectory of reprogramming intermediates defined by Thy1, SSEA1, and Oct4-GFP (OGFP) expression (Polo et al., 2012).

(B) Trajectories of reprogramming intermediates defined by CD44, ICAM1, and Nanog-GFP (NGFP) expression (O'Malley et al., 2013). Double lanes indicate transitions that occur at a higher frequency. iPSC, induced pluripotent stem cell; MEF, mouse embryonic fibroblast; MET, mesenchymal-epithelial transition; OSKM, Oct4, Sox2, Klf4, and c-Myc.

2012). This echoes previous reports that the mesenchymal-epithelial transition (MET) is a critical early event during induction of pluripotency in fibroblasts (Li et al., 2010; Samavarchi-Tehrani et al., 2010).

A caveat of this roadmap is that the great majority of SSEA1$^+$ cells never become iPSCs. In addition, this surface antigen is heterogeneously expressed in embryonic stem cells (ESCs) and iPSCs. An alternative roadmap to pluripotency was proposed based on the emergence of the surface marker ICAM1, which is uniformly expressed in pluripotent cells (O'Malley et al., 2013). However, this surface marker is also expressed in about 50% of fibroblasts. To discriminate between cells at early, intermediate, and late stages of reprogramming, this study also considered expression of the surface marker CD44 and a Nanog-GFP reporter (Figure 1B). Dox-mediated induction of OSKM resulted in the gradual disappearance of CD44$^+$/ICAM1$^-$ cells and appearance of CD44$^-$/ICAM1$^+$ cells around day 6. Notably, some Nanog-GFP$^+$ cells were detected even earlier. By sorting subpopulations on day 10 based on the expression of CD44, ICAM1, and Nanog, the authors predicted the efficiency of distinct transitions between these subpopulations. This revealed that few cells were capable of activating Nanog-GFP within a 24 h window. However, Nanog-GFP$^+$ cells had a superior capacity to generate iPSCs compared to Nanog-GFP$^-$ cells with the same CD44/ICAM1 profile. Thus, it was concluded that activation of Nanog is a rate-limiting step during iPSCs generation.

Expression profiling along the CD44/ICAM1/Nanog roadmap revealed two notable trends. First, a cohort of endogenous pluripotency genes (including *Oct4*, *Sall1*, and *Sall4*) was already significantly activated by the time cells had reached the early signposts marked by disappearance of CD44 expression or appearance of Nanog-GFP activity (Figure 1B). Second, O'Malley and colleagues observed a transient up- and downregulation of epidermal genes in intermediate states. This trend was also observed in prior expression analyses of partially reprogrammed cells (Mikkelsen et al., 2008; Sridharan et al., 2009), a time course analysis of bulk populations during reprogramming (Samavarchi-Tehrani et al., 2010), and the Thy1/SSEA1/Oct4 roadmap (Polo et al., 2012). These findings highlight gene expression dynamics that would not be expected a priori during the conversion of fibroblasts into iPSCs, revealing an unanticipated complexity to the process of epigenetic reprogramming.

An investigation of population-level chromatin dynamics during the initial stages of reprogramming reported the genome-wide redistribution of histone H3 lysine 4 dimethylation (H3K4me2), a mark associated with euchromatin, at thousands of loci (Koche et al., 2011). In comparison, little change was observed in the distribution of histone H3 lysine 27 trimethylation (H3K27me3), a transcriptional-silencing-associated marker, except for highly localized depletion at promoters that acquired histone H3 lysine 4 trimethylation (H3K4me3), an activation-associated marker. On the roadmap defined by Thy1/SSEA1/Oct4 status, fluctuations between activating and repressive histone modifications largely followed the observed "biphasic" change in transcriptional status (Polo et al., 2012). For example, silencing of fibroblast-specific genes was accompanied by acquisition of H3K27me3, whereas activation of pluripotency genes was accompanied by the loss of H3K27me3 and acquisition of H3K4me3. In agreement with the study of population-level chromatin dynamics (Koche et al., 2011), genes that were activated early already carried H3K4me3 marks in fibroblasts. In contrast, reorganization of the DNA methylation landscape was not observed until the later phase of reprogramming (Polo et al., 2012). In accordance, genes involved in DNA methylation and demethylation, such as the de novo methyltransferases *Dnmt3a* and *Dnmt3L*, *Apobec*, and the 5-methylcytosine hydroxylase *Tet1*, were not upregulated until after day 9.

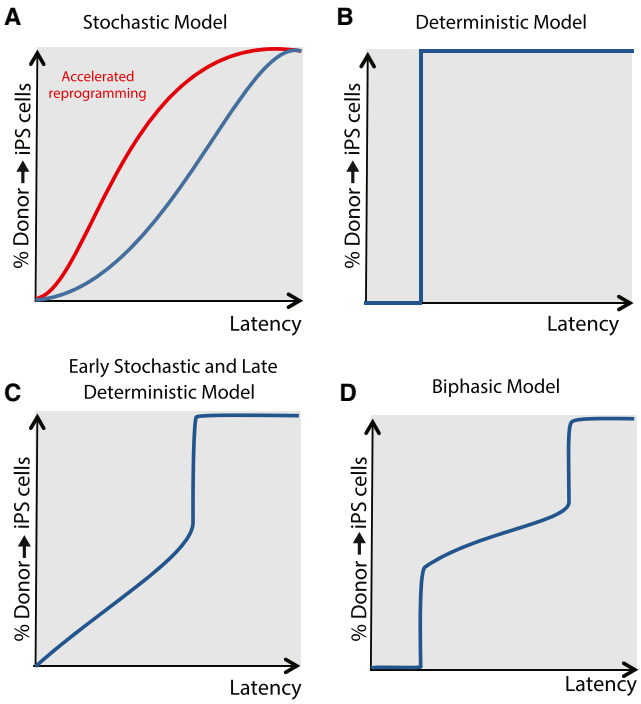


Figure 2. Models of Epigenetic Reprogramming

(A–D) Kinetics of reprogramming are graphically represented as a function of latency and the cumulative proportion of donor cells that gives rise to iPSCs. Latency indicates absolute time or the number of cell divisions. (A) Numerical modeling during OSKM-mediated reprogramming of secondary B cells demonstrated that induced pluripotency is essentially stochastic, but amenable to acceleration by cell-division-rate-dependent modifications such as overexpression of Lin28 or disruption of the p53/p21 pathway or a cell-division-rate-independent modification such as overexpression of Nanog (Hanna et al., 2009). (B) Deterministic model of reprogramming whereby somatic cells transit to pluripotency with a fixed latency. This type of reprogramming has only been observed by overexpression of C/EBPα (Di Stefano et al., 2013) or with the use of highly cycling donor cells (Guo et al., 2014). Deterministic reprogramming was also observed upon elimination of Mbd3 (Rais et al., 2013), but a recent study concluded that Mbd3 has a beneficial effect during the reprogramming process (dos Santos et al., 2014). (C) Model of reprogramming inferred from single-cell expression profiling (Buganim et al., 2012). (D) Biphasic model of reprogramming inferred from gene expression profiling along the Thy1/SSEA1/Oct4-GFP roadmap (Polo et al., 2012).

What do expression analyses tell us about the temporal requirements of individual reprogramming factors? c-Myc targets were predominantly regulated during the first wave of gene activity, while common targets of the Oct4-Sox2 complex were gradually activated later (Polo et al., 2012). This pattern corroborates a previous study that identified c-Myc as the predominant inducer of early changes in gene expression by expressing each factor individually in fibroblasts (Sridharan et al., 2009). Somewhat surprisingly, Klf4 targets were regulated both during the early and late phases (Polo et al., 2012). This finding suggests that, in addition to its established function in promoting the activation of the pluripotency network (Hall et al., 2009), Klf4 contributes actively to the early phase of reprogramming. It appears that this early role can be explained, at least in part, by promoting gene expression changes associated with MET. Bone morphogenetic proteins (BMPs), which also promote MET during the early phase of reprogramming (Samavarchi-Tehrani et al., 2010), could replace Klf4 and induce pluripotency in fibroblasts with Oct4 alone (Chen et al., 2011).

While both roadmaps provide enhanced resolution compared to bulk population analyses, it is important to bear in mind that neither study described markers that were fully capable of predicting the outcome of the reprogramming process. Hochedlinger and colleagues point out that the surface markers c-Kit, EpCAM, and PECAM1 become activated at successive time points in SSEA1$^+$ cells and may further enrich for intermediates with the potential to form iPSCs (Polo et al., 2012). However, it is likely that the expression of one or more transcriptional determinants of pluripotency will be needed to identify cells with the capacity to reprogram in a purely deterministic fashion. Quantitative analysis of gene expression in single cells (Fluidigm) and single-molecule RNA FISH techniques revealed that expression of *Esrrb*, *Utf1*, *Lin28*, or *Dppa2* is a better predictor of progression to pluripotency than previously suggested markers, including *Oct4* (Buganim et al., 2012). Thus, the identification of a truly predictive set of markers may require the use of novel reporter alleles. In this regard it is important to point out that reporter-based assessment of gene expression in pluripotent cells can be significantly influenced by the genetic background and gene-targeting strategy. For example, heterozygous loss-of-function knockin reporters do not faithfully reflect expression of Nanog in mouse ESCs (Faddah et al., 2013; Filipczyk et al., 2013). The use of self-cleaving fluorescent reporters downstream of coding sequences reduces the risk of altering endogenous transcriptional control mechanisms.

Several models have been proposed to explain the kinetics of reprogramming. The stochastic model holds that pluripotency is acquired as a result of the random occurrence of one or more rate-limiting steps. Consequently, iPSCs are generated with variable latencies (Figure 2A). In contrast, the deterministic model posits that reprogramming proceeds through a defined order of events with fixed latency (Figure 2B). Numerical modeling demonstrated that OSKM-mediated reprogramming is essentially stochastic, but amenable to acceleration by modifications such as inhibition of the p53/p21 pathway and overexpression of Lin28 or Nanog (Hanna et al., 2009). Analyses of single cells (Buganim et al., 2012) and intermediate subpopulations (Polo et al., 2012) indicated that distinct phases of the reprogramming process are associated with stochastic or deterministic changes in gene expression. The studies reached different conclusions regarding the timing of these phases: single-cell analysis revealed that early changes in gene expression are largely stochastic, while the later stages follow a deterministic order of events starting with activation of endogenous *Sox2* (Figure 2C) (Buganim et al., 2012). This deterministic phase of reprogramming appears to coincide with the stabilization phase described by Wrana and colleagues, during which repression of OSKM transgenes allows full expression of the pluripotency network (Golipour et al., 2012). On the other hand, the roadmap defined by Thy1/SSEA1/Oct4 expression identified two major waves of gene activity at the beginning and end of reprogramming, with a predominantly stochastic phase observed in between (Figure 2D) (Polo et al., 2012). As we discuss later, the efficiency of reprogramming, however, can be dramatically enhanced by the removal of specific barriers to induced pluripotency, challenging previous assumptions about the stochastic nature of reprogramming.

Drivers of Epigenetic Reprogramming: Mixing Up Yamanaka's Cocktail

Starting with a pool of 24 candidate factors, Yamanaka used a process of elimination to identify OSKM as the original reprogramming cocktail in mouse fibroblasts (Takahashi and Yamanaka, 2006). We now know that there is remarkable flexibility in the choice of defined factors that can induce pluripotency (Figure 3A). Here we reconstruct how Yamanaka's cocktail of defined factors has been modified over time and how this informs our understanding of the reprogramming process.

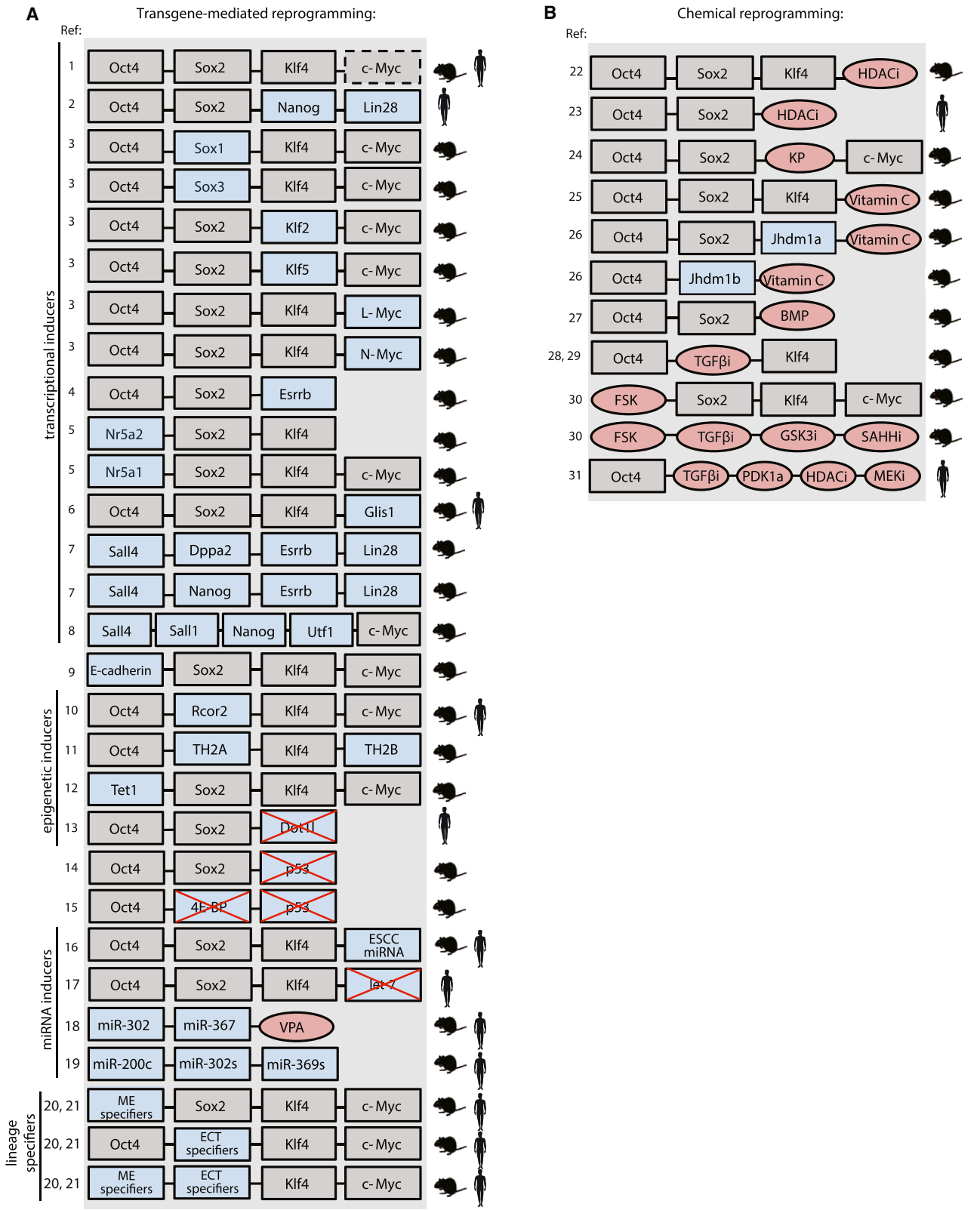
One of the first modifications was to reprogram fibroblasts into iPSCs using Oct4, Sox2, and Klf4 (OSK) alone (Nakagawa et al., 2008; Wernig et al., 2008). However, the three-factor reprogramming process was significantly delayed compared to cells also transduced with the oncogene c-Myc. In hindsight, including c-Myc was a stroke of genius that made the process sufficiently efficient to detect the first iPSCs in culture. The role of c-Myc remains a subject of debate, but its dominant role during the early phase of reprogramming is widely recognized (Polo et al., 2012; Sridharan et al., 2009). Compared to target genes of OSK, targets of c-Myc are strongly associated with an active chromatin signature in mouse ESCs (Kim et al., 2008). It is also known that c-Myc regulates transcriptional pause release at a third of all actively transcribed genes in ESCs (Rahl et al., 2010). Therefore, c-Myc may enhance the efficiency of iPSC generation by transcriptional amplification of genes involved in cellular proliferation. Zaret and colleagues reported that OSK act as pioneer factors for c-Myc at distal elements and promoters with closed chromatin and that binding by the reprogramming factors is not predominantly dictated by pre-existing open histone modifications (Soufi et al., 2012). This study assigned a direct role to c-Myc in facilitating the initial engagement of OSK with many chromatin sites.

Each of the original Yamanaka factors can be replaced by other transcription factors. This attribute has revealed a high degree of redundancy among the genetic factors capable of inducing pluripotency. As might be expected from their structural similarity, homologs of Klf4, Sox2, and c-Myc had reprogramming activity in mouse fibroblasts (Nakagawa et al., 2008). Klf4 and c-Myc were replaced by the combination of Nanog and Lin28 in human fibroblasts (Yu et al., 2007) and by the orphan nuclear receptor Esrrb in mouse fibroblasts (Feng et al., 2009). Nanog and Esrrb may operate through similar mechanisms as Esrrb is a direct transcriptional target of Nanog that, like Nanog, can maintain self-renewal of mouse ESCs in the absence of leukemia inhibitory factor (LIF) signaling (Festuccia et al., 2012). Another orphan receptor, Nr5a2, and to a lesser extent, Nr5a1, replaced the requirement for exogenous Oct4, but this likely occurs through activation of endogenous *Oct4* (Heng et al., 2010). Oct4 can also be replaced by E-cadherin, a master regulator of the epithelial phenotype (Redmer et al., 2011). The underlying mechanism is unclear, but E-cadherin overexpression prevents the nuclear localization of β-catenin, a negative regulator of the early phase of reprogramming (Ho et al., 2013).

The repertoire of transcription factors capable of inducing pluripotency was further expanded by single-cell expression analysis of 48 genes during reprogramming (Buganim et al., 2012). A Bayesian network was derived by monitoring multiple clonally related single sister cells at different time points. This model holds that activation of endogenous *Sox2* initiates a series of consecutive steps leading to the activation of many pluripotency genes. According to this analysis, Sox2 first turns on *Sall4*, which then activates four downstream targets, including *Oct4*. This hierarchical model of gene activation predicted combinations of transcription factors that did not include Oct4 or Sox2, but were capable of inducing pluripotency. Furthermore, this study identified two combinations of four factors that could replace OSKM entirely: Sall4, Esrrb, and Lin28 combined with either Dppa2 or Nanog (Buganim et al., 2012).

Methodologies to induce cell fate conversion typically use transcription factors, rather than chromatin-modifying enzymes, to initiate reprogramming. However, several studies have identified epigenetic regulators that can replace some of the Yamanaka factors, directly highlighting epigenetic remodeling events of functional importance for induced pluripotency. These regulators include the corepressor Rcor2 (Yang et al., 2011), the 5-methylcytosine (5mC) hydroxylase Tet1 (Gao et al., 2013), and the histone variants TH2A and TH2B (Shinagawa et al., 2014). In addition, some Yamanaka factors can be replaced by the removal of epigenetic barriers to reprogramming. For example, human fibroblasts can be reprogrammed by Oct4 and Sox2 alone after knockdown of DOT1L, the histone H3 lysine 79 (H3K79) methyltransferase (Onder et al., 2012). DOT1L inhibition facilitated the loss of H3K79 dimethylation (H3K79me2), an activation-associated marker, from fibroblast genes associated with the epithelial-to-mesenchymal transition (EMT). Furthermore, *DOT1L* was downregulated by the Toll-like receptor 3 (TLR3) pathway, which may facilitate reprogramming by stimulating the innate immune response (Lee et al., 2012).

A subset of the microRNA (miR)-*290* cluster, called the ESC-specific cell cycle (ESCC)-regulating miRNAs, enhanced the efficiency of reprogramming by OSK and may be a downstream target of c-Myc (Judson et al., 2009). Surprisingly, two groups reported that overexpression of some miRNAs can replace transcriptional inducers of pluripotency altogether (Anokye-Danso et al., 2011; Miyoshi et al., 2011). miRNAs promote reprogramming through multiple mechanisms, including the blocking of TGF-β-induced EMT and regulation of cell cycle-related genes (Liao et al., 2011; Subramanyam et al., 2011). In addition, the miRNAs *miR-205* and *miR-200* family members were implicated in the promotion of MET during the early phase of reprogramming in response to BMP signaling (Samavarchi-Tehrani et al., 2010). Yamanaka and colleagues demonstrated that some miRNAs have an opposite effect on the reprogramming process (Worringer et al., 2014). Specifically, this study reported that *let-7* miRNAs inhibit the expression of LIN-41, a translational repressor of the prodifferentiation gene EGR1. An antisense inhibitor of *let-7* increased the efficiency of OSK-induced reprogramming of human fibroblasts by one or two orders of magnitude, achieving an efficiency similar to that observed with OSKM. Finally, miRNAs can also serve as signposts of the reprogramming process, delineating alternative paths to induced pluripotency. Blelloch and colleagues targeted fluorescent reporters into the *miR-290* and *miR-302* clusters and tracked the activation of these two miRNAs during somatic cell reprogramming (Parchem et al., 2014). This analysis revealed that a cell's trajectory is dependent on the choice of reprogramming factors: whereas *miR-290* and *miR-302* were activated in a stochastic



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manner during OSK-induced reprogramming, these miRNA loci were activated sequentially when Sall4 was included in the cocktail of defined factors.

Most of the inducers of pluripotency discussed thus far are factors that are highly expressed in ESCs. Two studies reported that Oct4 and Sox2 could be replaced by lineage specifiers that are not enriched in ESCs. Deng and colleagues identified Gata3 as an unexpected Oct4 replacer by screening a plasmid library (Shu et al., 2013). Gene expression analysis showed that viral infection of Oct4 and Gata3 in fibroblasts inhibits the expression of a set of ectodermal specification (ECT)-related genes that are elevated by Sox2, Klf4, and c-Myc. The authors then investigated whether other mesendodermal (ME) lineage specifiers could induce a similar transcriptional response, identifying Gata6, Sox7, Pax1, Gata4, C/EBPα, HNF4a, and Grb2 as alternative Oct4 replacers. Sox2 and its reprogramming substitutes, Sox1, Sox3, and Gmn, attenuated the induction of ME genes after infection with Oct4, Klf4, and c-Myc. Remarkably, multiple combinations of ECT and ME specifiers were able to replace Oct4 and Sox2 simultaneously (Shu et al., 2013). Similar results were described during reprogramming of human fibroblasts to pluripotency (Montserrat et al., 2013). This finding led to the proposition of the ''seesaw'' model, which holds that a somatic cell has greater potential of reaching pluripotency when it is balanced by two opposing differentiation potentials.

Insights from Chemical Reprogramming

An important objective of current work in epigenetic reprogramming is to develop transgene-free methodologies for inducing pluripotency. These approaches lay the foundation for safer and more accessible reprogramming technologies that would be suitable for human therapeutic applications. Some of these studies have implicated previously unsuspected signaling pathways and epigenetic mechanisms in the induction of pluripotency, often after screening extensive collections of small molecules. Here we review the most promising chemical approaches to reprogramming and discuss the underlying mechanisms.

Initial reports described chemicals that facilitate the transition to pluripotency in stable reprogramming intermediates, including the DNA methyltransferase inhibitor 5-aza-cytidine (Mikkelsen et al., 2008) and the 2i cocktail, which consists of the MEK inhibitor PD0325901 and GSK3 inhibitor CHIR99021 (Silva et al., 2008). Several groups have shown that 2i induces global hypomethylation in pluripotent cells by induction of Prdm14, which in turn causes the downregulation of de novo DNA methyltransferases (see the Review in this issue by Lee et al. [2014]). These observations suggest that 5-aza-cytidine and 2i may promote the final stages of reprogramming through partially overlapping epigenomic mechanisms. Smith and colleagues have shown that GSK3 inhibition promotes the self-renewal of ESCs by removing the repressive influence of Tcf3 on the expression of *Esrrb* (Martello et al., 2012). Therefore, another plausible mechanism underlying the effect of GSK3 inhibition is the activation of Esrrb, a component of several reprogramming factor cocktails (Figure 3A) (Buganim et al., 2012; Feng et al., 2009).

Chemical screens have identified compounds that replace individual Yamanaka factors during iPSC generation (Figure 3B). Melton and colleagues reported that histone deacetylase (HDAC) inhibitors, such as valproic acid (VPA), enabled efficient reprogramming of mouse fibroblasts in the absence of c-Myc (Huangfu et al., 2008a) and human fibroblasts in the absence of both c-Myc and Klf4 (Huangfu et al., 2008b). A different screen identified the GSK3-β and CDK inhibitor kenpaullone (KP) as a replacer of Klf4 (Lyssiotis et al., 2009). However, its effect was not phenocopied by other GSK3-β and CDK inhibitors, indicating that KP has a different mode of action. Inhibition of TGF-β signaling was capable of activating endogenous *Nanog* and replacing the requirement for ectopic Sox2 and c-Myc (Ichida et al., 2009; Maherali and Hochedlinger, 2009). Since TGF-β signaling is activated during ME specification, this result lends further support to the hypothesis that Sox2 represses ME genes during reprogramming, a central tenet of the seesaw model (Montserrat et al., 2013; Shu et al., 2013).

Vitamin C enhanced the induction of pluripotency in murine and human fibroblasts (Esteban et al., 2010). The underlying mechanisms have been the focus of several studies. Vitamin C reduced histone 3 lysine 36 dimethylation and trimethylation (H3K36me2/3) levels by potentiating the demethylases Jhdm1a/1b (Wang et al., 2011). Specifically, Jhdm1b (also known as Kdm2b) suppressed cell senescence by removing H3K36me2/3 marks from the *Ink4/Arf* locus, resulting in its transcriptional silencing. In addition, the removal of H3K36me2/3 marks from the *miR-302/367* cluster by Jhdm1b facilitated binding and activation by Oct4. Thus, changes in this histone modification mark are associated with both gene activation and gene repression. The important role of these histone demethylases was underscored by the observation that forced expression of Jhdm1a replaced Klf4 and c-Myc, while Jhdm1b replaced Sox2, Klf4, and c-Myc in the presence of vitamin C (Wang et al., 2011). Jhdm1b was also shown to have an alternative, vitamin C-independent role by contributing to activation of early responsive genes during reprogramming through its H3K36 demethylase domain (Liang et al., 2012).

Loss of imprinting at the *Dlk1-Dio3* locus was reported to reduce the efficiency with which iPSCs contribute to ''all-iPSC'' mice by tetraploid (4n) complementation (Stadtfeld et al., 2010). Another study found, however, that competence to form all-iPSC mice was primarily dependent on the stoichiometry of reprogramming factor expression (Carey et al., 2011). Hochedlinger and colleagues reported that chemical treatment

Figure 3. Methodologies for Inducing Pluripotency

(A) Transgene-mediated reprogramming strategies: [1]Takahashi and Yamanaka, 2006; [2]Yu et al., 2007; [3]Nakagawa et al., 2008; [4]Feng et al., 2009; [5]Heng et al., 2010; [6]Maekawa et al., 2011; [7]Buganim et al., 2012; [8]Mansour et al., 2012; [9]Redmer et al., 2011; [10]Yang et al., 2011; [11]Shinagawa et al., 2014; [12]Gao et al., 2013; [13]Onder et al., 2012; [14]Kawamura et al., 2009; [15]Tahmasebi et al., 2014; [16]Judson et al., 2009; [17]Worringer et al., 2014; [18]Anokye-Danso et al., 2011; [19]Miyoshi et al., 2011; [20]Shu et al., 2013; [21]Montserrat et al., 2013.

B) Chemical reprogramming strategies: [22]Huangfu et al., 2008a; [23]Huangfu et al., 2008b; [24]Lyssiotis et al., 2009; [25]Esteban et al., 2010; [26]Wang et al., 2011; [27]Chen et al., 2011; [28]Maherali and Hochedlinger, 2009; [29]Ichida et al., 2009; [30]Hou et al., 2013; [31]Zhu et al., 2010. Original Yamanaka factors are colored light gray. This is not an exhaustive list of factor and chemical combinations, but only includes those methods highlighted in the text. Note that these studies were mainly performed in fibroblasts, and transgene requirements may be different for other types of somatic donor cells, such as neural stem cells.

may also improve the quality of iPSCs. Vitamin C enabled the reprogramming of B lymphocytes into iPSCs with 4n capability (Stadtfeld et al., 2012). The effect of vitamin C was attributed to preserved imprinting in the *Dlk-Dio3* locus by the maintenance of H3K4me2 and acquisition of H3K4me3. These histone modifications prevented binding of Dnmt3a, which is essential for *Dlk1-Dio3* DNA hypermethylation but can only recognize unmodified H3K4 tails.

Combined inhibition of intracellular signaling and epigenetic remodeling enables the replacement of multiple Yamanaka factors simultaneously. However, replacement of Oct4 long remained a stumbling block on the road toward chemically induced pluripotent cells. Deng and colleagues performed high-throughput chemical screening to successfully identify several Oct4 replacers, including the cyclic AMP agonist forskolin (FSK) (Hou et al., 2013). When FSK was used in conjunction with a cocktail of inhibitors previously reported to support Oct4-induced reprogramming, the authors were able to derive germline-competent iPSCs without the use of transgenes. Four essential small molecules were identified: FSK, the GSK3 inhibitor CHIR990291, the TGF-β inhibitor 616452, and DZNep, an *S*-adenosylhomocysteine (SAH) hydrolase inhibitor. While the mechanisms underlying this chemical reprogramming method remain incompletely understood, it is clear that these compounds activate several inducers of pluripotency. In particular, chemical treatment stimulated the expression of *Gata4*, *Gata6*, *Sall4*, *Sox2*, and *Sox17*. The inclusion during the final stages of reprogramming of DZNep, which represses *S*-adenosylmethionine (SAM)-dependent cellular methylation events, decreased DNA methylation and H3K9 methylation levels at the *Oct4* promoter (Hou et al., 2013).

So far these conditions have not been successfully applied to the chemical reprogramming of human somatic cells into iPSCs. However, some progress has been reported with chemical reprogramming in human cells. OCT4-induced reprogramming of human primary somatic cells was achieved by combining TGF-β inhibition, HDAC inhibition, and MEK inhibition with a small molecule activator of 3′-phosphoinositide-dependent kinase-1 (PDK1) (Zhu et al., 2010). Therefore, the successful generation of chemically induced, patient-specific iPSCs may be contingent on identifying druggable activators of *OCT4* in human cells.

Inducing Novel States of Pluripotency

Human ESCs and iPSCs exhibit molecular and biological properties similar to epiblast stem cells (EpiSCs) derived from the mouse postimplantation epiblast. These cells express pluripotency genes such as *Oct4*, *Sox2*, and *Nanog*, but only rarely contribute to chimeric mice after blastocyst injection (Brons et al., 2007; Tesar et al., 2007). This limitation has led to the suggestion that EpiSCs represent a "primed" state of pluripotency, as opposed to the "naive" state of mouse ESCs (Nichols and Smith, 2009). An important aim of current research is to define the state of pluripotency and to assess whether human ESCs can be induced into the naive state. Several studies described small molecules that induce a naive-like state in conventional human ESCs. Hanna and colleagues reported that a medium comprising inhibitors of Jun kinase and p38 MAP kinase in addition to 2i, LIF, FGF, and TGF-β induces naive properties in human ESCs (Gafni et al., 2013). These cells had a globally altered chromatin profile, including reduced levels of H3K27me3 at developmental genes and a global reduction in poised enhancers marked by the presence of H3K4me1 and H3K27me3 and the absence of H3K27ac. Remarkably, human iPSCs cultured under these conditions made a contribution to chimeric embryos after injection into mouse morulae. Another study described the isolation of naive-like human ESCs by culture in mTeSR1 medium, which contains high levels of FGF, supplemented with PD0325901, the GSK3 inhibitor BIO, LIF, and Dorsomorphin, an inhibitor of BMP signaling (Chan et al., 2013). Under these conditions OCT4 and NANOG occupied a significantly different set of target genes. In addition, derivation of human ESCs in 2i and FGF generated cells with a similar expression profile as previously reported transgene-dependent naive-like human ESCs (Ware et al., 2014). Surprisingly, all of these studies observed a requirement for FGF, which in the mouse system stimulates differentiation into the primed state. Additional pathways involved in the establishment and maintenance of naive human pluripotency may yet be identified through high-throughput chemical screening. It will also be of interest to explore whether human ESCs display a dynamic equilibrium between naive and primed states, as has been described by Surani and colleagues in single mouse ESCs based on the heterogeneous expression of *Stella* (Hayashi et al., 2008).

A recent study suggested that exposure of murine somatic cells to stress, such as low pH, may induce a novel state of pluripotency with the potential to contribute to both embryonic and placental tissues (Obokata et al., 2014). This work has received intense scrutiny in recent months due to lack of reproducibility. However, an expanded fate potential was previously observed in subpopulations of mouse ESCs. Pfaff and colleagues reported that ESCs fluctuate through a totipotent "two-cell-like" state, which is characterized by global enrichment in activation-associated histone marks and hypomethylation at endogenous retroviral (ERV) elements (Macfarlan et al., 2012). In addition, Brickman and colleagues described heterogeneous expression of the extraembryonic endoderm marker *Hex* in mouse ESCs cultured in 2i + LIF. Purified *Hex*-positive ESCs contributed efficiently to both the epiblast and all extraembryonic lineages following morula aggregation (Morgani et al., 2013). This expanded differentiation potential was also observed upon injection of single *Hex*-positive ESCs into morulae, providing evidence of totipotency at the single-cell level. Unlike two-cell-like ESCs marked by ERV expression, *Hex*-positive ESCs continued to express core pluripotency genes, including *Oct4*, *Sox2*, and *Nanog*. Future experimental work is needed to examine conclusively whether totipotent cells can be induced directly from somatic cells through environmental stimuli, chemical manipulation, or other means.

The question of whether iPSCs retain characteristics of their somatic origin has been a subject of considerable interest. While low-passage iPSCs may exhibit some epigenetic memory (Kim et al., 2010), extended passaging brought the methylation patterns of iPSCs closer to ESCs (Nishino et al., 2011; Polo et al., 2010). In addition, the effect of subtle variations in culture methods between laboratories appears to overshadow any consistent gene expression differences between human iPSCs and ESCs (Newman and Cooper, 2010), which also display little

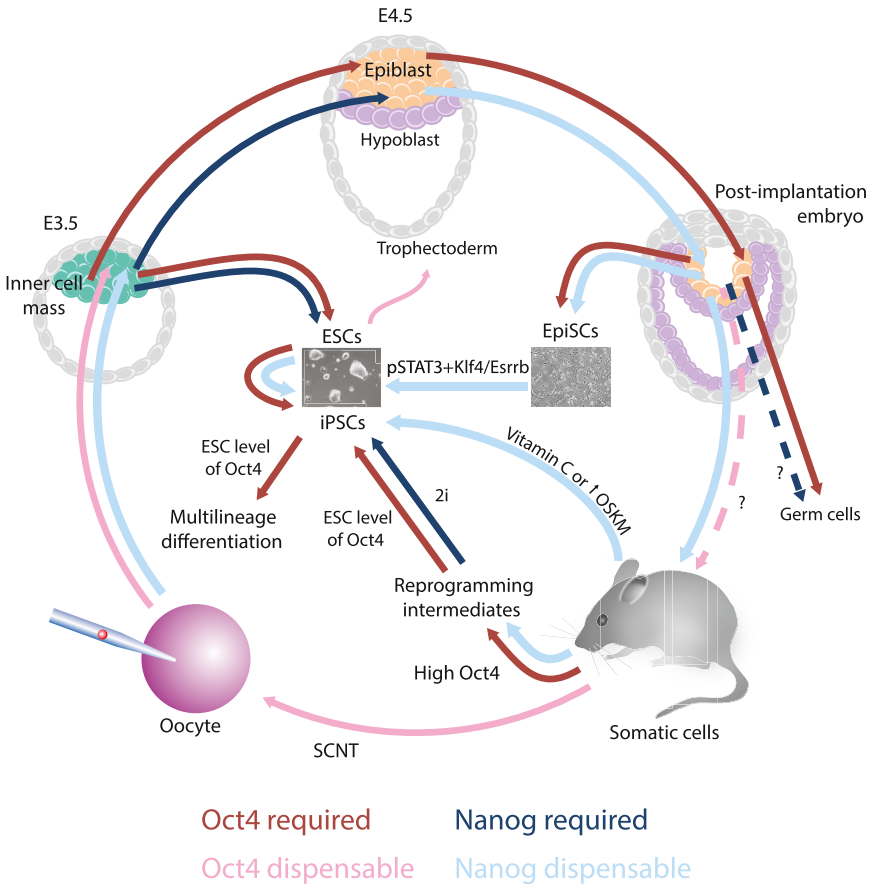

Figure 4. Gatekeepers of Pluripotency
Requirements of the master transcription factors Oct4 and Nanog during epigenetic reprogramming events in the mouse system in vitro and in vivo. Broken lines indicate cell fate transitions where the role of Oct4 or Nanog is contested (see text for details). ESCs, embryonic stem cells; EpiSCs, epiblast stem cells; SCNT, somatic cell nuclear transfer; 2i, cocktail of MEK and GSK3 inhibitors.

difference in terms of the global distribution of H3K4me3 and H3K27me3 (Guenther et al., 2010). We conclude that reprogramming with the Yamanaka factors under conventional conditions induces a state of pluripotency that is indistinguishable from ESCs for all practical purposes.

Gatekeepers of Pluripotency

While "drivers" of epigenetic reprogramming have received the lion's share of research interest, an important function is performed by endogenous master transcription factors whose upregulation controls entry to the pluripotent state. The best-characterized "gatekeepers" of pluripotency also serve as potent inducers or facilitators of reprogramming when ectopically expressed. However, the effects of overexpressing a transcription factor in fibroblasts must be discriminated from the role of its endogenous gene product during the later stages of iPSC induction. Loss-of-function experiments have traced the genetic requirements of two central pluripotency determinants, Oct4 and Nanog, during epigenetic reprogramming events in vivo and in vitro (Figure 4).

The POU family transcription factor Oct4 is required for the formation of pluripotent cells in the mouse embryo and the derivation of ESCs (Nichols et al., 1998). Oct4 is also continuously expressed throughout primordial germ cell (PGC) specification, and conditional deletion of Oct4 in PGCs caused apoptosis as early as E10 (Kehler et al., 2004). Early studies in mouse ESCs indicated that the dosage of Oct4 tightly controls cell fate as loss of Oct4 caused differentiation to the trophectoderm lineage, while elevated Oct4 expression resulted in differentiation into cells expressing endoderm and mesoderm markers (Niwa et al., 2000). In addition, a reduced expression level of exogenous Oct4 arising from subtle differences in polycistronic vector design affected the quality of iPSCs (Carey et al., 2011).

To further flesh out the dose-dependent role of Oct4 during induced pluripotency, Silva and colleagues reprogrammed *Oct4*$^{-/-}$ somatic cells with a piggyBac vector containing bicistronic Oct4 and Cherry sequences (Radzisheuskaya et al., 2013). This revealed that transition through the initial stages of reprogramming involved a range of Oct4 expression levels, while the resulting iPSCs invariably exhibited an ESC-level of Oct4. Consistent with this observation, high levels of OSKM transgene expression were detrimental during the final stage of reprogramming (Golipour et al., 2012). Once pluripotency was established, however, a 7-fold reduction in Oct4 expression did not cause the loss of self-renewal or downregulation of other core regulators, such as *Nanog*. In fact, Oct4-low iPSCs exhibited reduced responsiveness to differentiation-inducing signals (Radzisheuskaya et al., 2013), which was also observed in *Oct4*$^{+/-}$ ESCs (Karwacki-Neisius et al., 2013). Biochemically, reduced expression of Oct4 was associated with enhanced LIF sensitivity, upregulation of Wnt signaling ligands, and increased binding of Nanog to key target genes. Remarkably, differentiation into all three embryonic lineages was observed with iPSCs that constitutively expressed an ESC-level of Oct4, but not a reduced level of Oct4 (Radzisheuskaya et al., 2013). This finding suggests that Oct4 has a previously unappreciated role in cellular differentiation.

Do other methods for inducing epigenetic reprogramming also require Oct4? Surprisingly, oocytes deficient in maternal Oct4 were fully capable of reprogramming somatic cells by nuclear transfer (Wu et al., 2013). Furthermore, embryos deficient in both maternal and zygotic Oct4 still initiated lineage segregation between trophectoderm and the inner cell mass (ICM) at E3.5, but could not support the subsequent development of a viable naive pluripotent epiblast (Wu et al., 2013). Hence, genetic studies suggest there are distinct requirements for Oct4 during the establishment of totipotent and pluripotent cells.

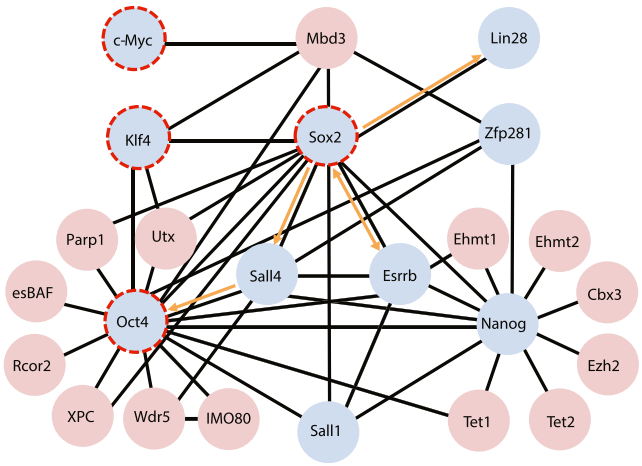

Figure 5. An Interactome of Reprogramming Factors
Protein-protein interactions between reprogramming factors (blue) and epigenetic modifiers (red) implicated in the induction of pluripotency. Red borders indicate the original Yamanaka factors. Interaction data was curated from interactome studies in mouse ESCs (Costa et al., 2013; Ding et al., 2012; Gagliardi et al., 2013; Gao et al., 2012; Wang et al., 2006) and additional studies described in the text. Superimposed on the interactome are regulatory relationships inferred from single-cell analysis during reprogramming (yellow arrows) (Buganim et al., 2012).

Like Oct4, the homeodomain transcription factor Nanog is required for the establishment of the naive pluripotent epiblast in vivo and the derivation of ESCs (Chambers et al., 2003; Mitsui et al., 2003; Silva et al., 2009). However, the role of Nanog in maintenance of pluripotent cells was redefined by the observation that it could be permanently removed from ESCs without compromising their ability to contribute to chimeric mice (Chambers et al., 2007). Nevertheless, *Nanog*$^{-/-}$ cells failed to develop into germ cells beyond E11.5. This study also reported that Nanog expression fluctuates, thereby predisposing a subset of ESCs to differentiation. However, single-cell analysis showed that expression of Nanog is equally variable as that of other pluripotency-associated genes (Faddah et al., 2013; Filipczyk et al., 2013). Unlike Oct4, Nanog was dispensable for the derivation and maintenance of EpiSCs (Osorno et al., 2012). In addition, the absence of a hypoblast in *Nanog*$^{-/-}$ blastocysts was attributed to a non-cell-autonomous requirement for paracrine support from the epiblast (Messerschmidt and Kemler, 2010). Thus, the requirement of Nanog appears to be restricted to the establishment of naive pluripotency and germ cell development. Both of these developmental transitions are associated with widespread epigenetic remodeling events. In female embryos, Nanog expression at E4.5 correlated precisely with the subset of cells that show X chromosome reactivation (Silva et al., 2009). Together with Oct4 and Sox2, Nanog was implicated in the repression of the *cis*-acting, noncoding RNA *Xist* (Navarro et al., 2008), suggesting that core pluripotency factors directly contribute to X chromosome reactivation.

While Nanog was not included in Yamanaka's original cocktail of reprogramming factors, its potent effects during induced pluripotency are widely documented. Nanog was used to reprogram human somatic cells to pluripotency (Yu et al., 2007) and was present in several factor combinations that replaced the Yamanaka factors (Buganim et al., 2012). In addition, overexpression of Nanog enhanced the efficiency of OSKM-mediated reprogramming (Hanna et al., 2009; Theunissen et al., 2011). Endogenous *Nanog* was dispensable during the initial stages of reprogramming, but required for establishment of pluripotency in 2i conditions (Silva et al., 2009). Recent studies demonstrated that the requirement of Nanog can be bypassed by exogenous provision of its downstream effectors, phosphorylated (p)-STAT3 and Klf4 or Esrrb (Festuccia et al., 2012; Stuart et al., 2014). This underscores the crucial role of LIF-STAT3 signaling during iPSC generation. In addition, vitamin C treatment enabled the induction of pluripotency in *Nanog*$^{-/-}$ somatic cells (Schwarz et al., 2014), which puts a spotlight on the role of Nanog in recruiting vitamin C-dependent Tet dioxygenases to core pluripotency loci (Chen et al., 2013a; Costa et al., 2013). Surprisingly, Eggan and colleagues reported that *Nanog*$^{-/-}$ iPSCs generated at low efficiency with high titers of OSKM made a contribution to the germline in chimeras produced by blastocyst injection (Carter et al., 2014). Future studies will need to resolve the precise extent of germline transmission in the absence of Nanog and address the discrepancy with observations made using *Nanog*$^{-/-}$ ESCs (Chambers et al., 2007). Another point of interest will be to investigate X chromosome dynamics during Nanog-independent reprogramming.

The intimate role of Oct4 and Nanog in the establishment of pluripotency has prompted interest in identifying their mechanisms of action. Acute depletion of each factor in mouse ESCs caused expression changes in a wide array of target genes, demonstrating that Oct4 and Nanog have a dual role in gene activation and repression (Loh et al., 2006). As we discuss below, these "gatekeepers" of pluripotency serve as hubs in transcription factor networks in ESCs, engaging with multiple different epigenetic regulators. The importance of these cofactors is underscored by the observation that a conserved linker region, which serves as an interface for protein-protein interactions with key epigenetic modifiers, was critical for the reprogramming activity of Oct4 (Esch et al., 2013).

Modulators of Epigenetic Reprogramming

Perturbation of various epigenetic regulators influences the kinetics of reprogramming. Many of these regulators are directly recruited by the reprogramming factors to stimulate the expression of core pluripotency genes. A network of published protein-protein interactions between factors implicated during iPSC generation reveals extensive interconnectivity between "drivers" and "modulators" of epigenetic reprogramming (Figure 5).

Chromatin remodelers are key components of the interactome of reprogramming factors. Combined overexpression of ESC-specific BAF (esBAF) components Smarca4/Brg1 and Smarcc1/BAF155 synergistically enhanced OSK-induced reprogramming of fibroblasts (Singhal et al., 2010). Mechanistically, these remodelers enhanced Oct4 binding to the *Sall4*, *Tcf3*, and *Dppa4* promoters and increased the activation-associated markers H3K4me3 and H3K9 acetylation (H3K9ac) on Oct4 target genes. Both Brg1 and BAF155 form a direct physical interaction with Oct4 (Ding et al., 2012), and the interaction with Brg1 was mapped to the evolutionarily conserved Oct4 linker region (Esch et al., 2013). A recent study demonstrated that binding

of INO80, the chromatin-remodeling ATPase, can distinguish actively expressed target genes from those repressed by the master transcription factors in mouse ESCs (Wang et al., 2014). Upon recruitment by Oct4 and the H3K4 methyltransferase complex component Wdr5, INO80 maintains nucleosome-depleted regions and recruits Mediator and RNA Polymerase II. Knockdown of INO80 reduced the efficiency of reprogramming, while injection of short interfering RNAs against INO80 in the one-cell embryo impaired blastocyst formation ex vivo. This study illustrates how a chromatin remodeler can coordinate input from Oct4 and a histone methyltransferase to stimulate pluripotency gene expression.

The role of Mbd3, the scaffold protein of the nucleosome remodeling and deacetylase (NuRD) complex, has received significant interest. Hanna and colleagues reported that depletion of Mbd3 in mouse and human somatic cells enhanced the efficiency of reprogramming to a deterministic level (Figure 2B) (Rais et al., 2013). Mbd3 physically interacts with all four OSKM factors, suggesting that recruitment of Mbd3 to downstream OSKM target genes may provide a ''brake'' on their reprogramming activity. However, treatment of human fibroblasts with short hairpin RNAs (shRNAs) against *MBD3* prior to OSKM expression did not improve the efficiency of reprogramming (Onder et al., 2012). More recently, Silva and colleagues reported that genetic or siRNA-mediated depletion of Mbd3 had no apparent effect during reprogramming of mouse fibroblasts, and in fact reduced the efficiency of reprogramming in neural stem cells (dos Santos et al., 2014). Furthermore, overexpression of Mbd3/NuRD enhanced the conversion of EpiSCs to naive pluripotency in conjunction with Nanog. In contrast to this work, Rais et al. (2013) used lentiviral reprogramming vectors and secondary somatic donor cells. While such technical and procedural differences may account for subtle variation, the wide discrepancy in phenotypic outcome between these studies is unexpected. It should be noted that deterministic reprogramming was also observed upon OSKM activation in a subset of fast-cycling bone marrow cells (Guo et al., 2014) or transient overexpression of C/EBPα followed by OSKM activation in primary B cells (Di Stefano et al., 2014). These studies do not exclude the possibility that deterministic reprogramming may be preceded by a short stochastic phase. It is likely, however, that the largely stochastic nature of Yamanaka's original protocol is not inherent to the process of induced pluripotency.

Histone methyltransferases and demethylases occupy a central role in the control of induced pluripotency. Wdr5, a core component of the Trithorax complex, was recruited by Oct4 to mediate trimethylation of H3K4 at loci of pluripotency genes in mouse ESCs (Ang et al., 2011). Knockdown of Wdr5 during the initial stages of OSKM-mediated reprogramming reduced the efficiency of iPSC generation, suggesting that Oct4 recruits the Trithorax complex to reconfigure the H3K4me3 signature in somatic cells. Hanna and colleagues demonstrated that Oct4, Sox2, and Klf4 engage with the histone demethylase Utx to remove the transcriptional-silencing-associated H3K27me3 mark from target loci including *Sall4*, *Sall1*, and *Utf1* (Mansour et al., 2012). Overexpression of these target genes together with c-Myc and Nanog provided an alternative to exogenous OSK expression for induction of pluripotency in fibroblasts (Figure 3A). B cells deficient in *Utx* acquired pluripotency with significantly reduced efficiency, as did primary human fibroblasts treated with short hairpin RNAs (shRNAs) against *UTX*. Therefore, transcriptional inducers of reprogramming can associate with both writers and erasers of histone marks to promote the expression of downstream targets.

Several modulators of reprogramming converge on regulation of the heterochromatic histone mark, H3K9 trimethylation (H3K9me3). Megabase-scale H3K9me3-containing regions of the genome were found to be refractory to OSKM binding in human fibroblasts (Soufi et al., 2012). In addition, H3K9 methylation at core pluripotency loci was identified as a major roadblock that prevents murine reprogramming intermediates from reaching the pluripotent state (Chen et al., 2013b; Sridharan et al., 2013). Global H3K9me3 levels during reprogramming are the outcome of a tug-of-war between vitamin C-dependent H3K9 demethylases and H3K9 methyltransferases, which are activated by BMPs in serum (Chen et al., 2013b). Plath and colleagues showed that inhibition of Cbx3, a reader of H3K9me3, enhanced the efficiency of reprogramming (Sridharan et al., 2013). The mechanism involves the repression of *Nanog* by the combined action of Cbx3 and the H3K9 methyltransferases Ehmt1, Ehmt2, and Setdb1. Intriguingly, Cbx3, Ehmt1, and Ehmt2 were all detected as part of Nanog protein complexes in mouse ESCs (Gagliardi et al., 2013). This overlap suggests that recruitment of H3K9 methyltransferases may constitute a constraint on the activity of Nanog, similar to recruitment of the transcriptional corepressor Zfp281, which mediates Nanog autorepression (Fidalgo et al., 2012).

Demethylation of pluripotency-associated promoter regions represents a critical epigenetic event during somatic cell reprogramming (Mikkelsen et al., 2008) and is thought to take place after transcriptional and histone modification changes (Polo et al., 2012) (Figure 1A). While DNA demethylation can occur through a passive mechanism involving the gradual loss of methylation by the maintenance DNA methyltransferase Dnmt1 in the course of cell division, active mechanisms for demethylation have been implicated in the reprogramming process. For example, activation-induced cytidine deaminase (AID) mediates deamination of 5mC to thymine, which is subject to DNA repair, resulting in cytosine exchange and demethylation. Knockdown of AID was used to show that the enzyme is required for initiation of nuclear reprogramming by cell fusion in the absence of DNA replication (Bhutani et al., 2010). However, other studies reported a requirement for DNA replication during fusion-mediated reprogramming (Foshay et al., 2012; Tsubouchi et al., 2013), suggesting that passive mechanisms are involved instead. In addition, it has been shown that *AID* deficiency destabilizes late but not early events during iPSC formation (Kumar et al., 2013) or is even fully dispensable (Shimamoto et al., 2014). Thus, the role of AID in epigenetic reprogramming remains unresolved.

Another possible mechanism for active DNA demethylation implicated during induced pluripotency is the conversion of 5mC into 5-hydroxymethylcytosine (5hmC), which may subsequently be converted into cytosine through mechanisms involving the base excision repair pathway. The ten-eleven translocation (Tet) family methylcytosine hydroxylases Tet1 and Tet2 were shown to form a physical interaction with Nanog, and coexpression of Nanog with either Tet1 or Tet2 enhanced the efficiency of reprogramming (Costa et al., 2013). Nanog-dependent

recruitment of Tet1 to key target genes, including *Esrrb* and *Oct4*, promoted conversion of 5mC into 5hmC. Further support for the role of Tet enzymes comes from reports that Tet1 can replace Oct4 during reprogramming (Gao et al., 2013), while genetic ablation of *Tet1*, *Tet2*, and *Tet3* blocked reprogramming in fibroblasts by interfering with the demethylation and reactivation of miRNAs required for MET (Hu et al., 2014). Pei and colleagues demonstrated that, in the presence of vitamin C, Tet1 actually behaves as a barrier to reprogramming by inhibiting MET (Chen et al., 2013a). On the other hand, Tet2 had a constitutively positive effect at all levels of vitamin C. Tet2 was also implicated in the establishment of a permissive chromatin signature at the *Nanog* and *Esrrb* loci during the early stage of reprogramming (Doege et al., 2012). In this context Tet2 cooperated with poly(ADP-ribose) polymerase 1 (Parp1), a nuclear protein involved in DNA damage repair, to control the levels of 5mC and 5hmC. Another family of DNA repair proteins involved in reprogramming are members of the XPC nucleotide excision repair complex, which is recruited by Oct4 and Sox2 to the *Nanog* and *Oct4* promoters (Fong et al., 2011).

The above examples illustrate how various modulators of induced pluripotency are recruited to specific DNA targets by transcriptional "drivers" of reprogramming. However, some epigenetic regulators have globally adverse or beneficial consequences for the pluripotent state. An example of an epigenetic modifier with a globally adverse role is the histone variant macroH2A, whose removal enhanced the efficiency of reprogramming (Gaspar-Maia et al., 2013; Pasque et al., 2012). MacroH2A is depleted in pluripotent cells, and its deposition is thought to repress pluripotency factors in differentiated cells. MacroH2A isoforms were particularly enriched at target genes of Utx, which are reactivated early during reprogramming (Gaspar-Maia et al., 2013). Conversely, an epigenetic modification implicated in promoting the final stages of induced pluripotency is citrullination of arginine residues mediated by the peptidylarginine deiminase Padi4 (Christophorou et al., 2014). This enzyme was upregulated during reprogramming and disrupted the binding of histone H1 to nucleosomal DNA, contributing to chromatin decondensation in pluripotent cells.

Finally, even manipulation of the general transcriptional and translational apparatus can influence the kinetics of reprogramming. Timmers and colleagues reported that high levels of TFIID, which is central to transcription initiation by RNA polymerase II, are essential for reprogramming and the self-renewal of pluripotent cells (Pijnappel et al., 2013). A plausible explanation for the selective TFIID dependency observed in the pluripotent state is that promoter sequences of core pluripotency genes have reduced affinity for TFIID. Similarly, depletion of eukaryotic translation initiation factor 4E (eIF4E) binding proteins (4E-BPs), which are translational repressors, reduced the efficiency of somatic cell reprogramming in part through enhanced translation of p21 mRNA (Tahmasebi et al., 2014). As p53 inhibits reprogramming by stimulating p21 transcription, the authors reasoned that loss of p53 would rescue the reprogramming deficit caused by the loss of 4E-BPs. In fact, depletion of 4E-BPs in $p53^{-/-}$ fibroblasts resulted in increased reprogramming compared to wild-type fibroblasts. This effect was explained by reduced transcription of p21 and higher levels of Sox2 and c-Myc, whose translation is eIF4E dependent. Accordingly, expression of exogenous Oct4 alone was sufficient to induce pluripotency in $p53^{-/-}$;*4E-BP1/2*$^{-/-}$ fibroblasts, providing yet another variation on Yamanaka's reprogramming cocktail (Figure 3A).

Concluding Remarks

Recent developments in epigenetic reprogramming require us to reconsider long-held assumptions about the process of induced pluripotency. iPSC generation with the Yamanaka factors was thought to be inherently a stochastic process, but this view has been challenged by observations of deterministic reprogramming using additional genetic manipulation or different populations of donor cells. The question of stochasticity is symptomatic of a broader challenge for research into the molecular mechanisms of epigenetic reprogramming. As novel methods to induce pluripotency continue to be discovered, past assumptions about the parameters affecting reprogramming must be re-evaluated. Only a comparative approach can distinguish essential regulators and mechanisms from the idiosyncrasies of individual reprogramming methods. For this reason it will be especially illuminating to compare the sequence of events during chemical reprogramming or nuclear transfer with the roadmaps of transcription factor-induced reprogramming described above.

A second major aim of future experimental work will be to define crosstalk among the numerous molecular agents implicated in the reprogramming process. The growing repertoire of transcription factors capable of generating iPSC has repudiated the concept, once prevalent, of a pluripotent state governed by a triad of master transcription factors. Further complexity arises from long-range chromosomal interactions that connect several pluripotency-associated loci (Apostolou and Hochedlinger, 2013). In addition, high-throughput chemical screens and proteomic studies have identified a spectrum of chromatin remodelers, histone modifiers, DNA repair proteins, and 5mC hydroxylases that alter the kinetics of reprogramming. Many of these enzymes were implicated in the transcriptional regulation of core pluripotency genes. However, regulatory interactions between the upstream regulators themselves remain incompletely understood. The litmus test for any systems-level model of epigenetic reprogramming will be to establish a functional hierarchy among these factors.

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